7.0 TOXICITY TESTING REQUIREMENTS

This section provides a description of the surface water toxicity testing protocol that will be implemented during Phase IIA. Detailed SOPs will be provided by the toxicity testing laboratory.

Test Waters

One large volume (about 200 L) of site water containing LA will be provided to the toxicity testing laboratory by EPA. This water will be stored in the dark at 1-4°C prior to use in the tests in order to minimize the growth of algae or any other biological organisms.

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This water will be tested for toxicity to fish at the following dilutions assuming site water contains approximately 100Mf/L:

100% (undiluted)
10%
1%
0.1%
0.01%
0.001%
0%

The dilutions shall be prepared using reconstituted laboratory water at a hardness and pH that approximates site water in accord with Section 7.2.3 of EPA (2002). As indicated above, reconstituted laboratory water with no added site water will also be tested to serve as a reference water.

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Comment [DW1]: I don't know if this reference is viable with this modification but I think it is important to try to match this..Hardnesses from phase 1 were a little high (250-300).

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Water Chemistry

The concentration of LA, other contaminants of potential concern (e.g., metals) and selected water quality parameters (e.g., hardness, pH) is this water will be measured by EPA.

All test waters (including reference water) will be monitored in the laboratory for the following parameters:

Parameter	Frequency
Temperature	Daily
pH	Daily
Dissolved oxygen	Once per 5 days before swimup, and then at the start and end of each static
Ammonia	

	renewal (every 3 days)

Test Species

The test species will be rainbow trout (*Oncorhynchus mykiss*)

Life Stage

The life stage will be newly hatched larvae.

Exposure Conditions

Exposure will be performed using a static renewal protocol in 4-Liter aquaria. The renewal water must be thoroughly mixed each time before drawing samples for replacement of aquaria waters.

There will be 15 larvae per aquarium, with three aquaria per test water (a total of 45 larvae per test water).

Water temperature of test aquaria will be maintained at 12 ±1°C.

Exposure duration will be 6 weeks (42 days).

During the larval stage, water will be changed once every 10 days.

Swim-up is expected to occur on or about day 20 (after about 240 degree-days). After swim-up occurs, water will be changed once every three days. Based on this design, the total volume of water required for each test is approximately 150 L.

In order to ensure that fiber settling does not occur, each aquarium will be equipped with an air bubbler placed in the bottom and run continuously. In addition, each aquarium will be equipped with a circulating filter, used without any filter media.

PRELIMINARY TEST OF MIXING

In order to confirm that aeration and circulation are sufficient to keep LA fibers suspended in the aquaria, the following procedure will be performed once before the tests with fish are begun:

1. Place a site test water containing LA into one 4-L aquarium and establish aeration and circulation conditions exactly as will be done when fish are present.

2. After 3 days, remove approximately 100 mL of water from the tank at a depth of about 1-2 cm below the surface of the water. Place the sample into a clean plastic bottle. Label this bottle "LA Fiber Suspension Test: Sample A".
3. Remove approximately 100 mL midway between the top and the bottom of the tank and place this sample into a second clean plastic bottle labeled "LA Fiber Suspension Test: Sample B".
4. Remove approximately 100 mL from a depth of 0-2 cm above the bottom of the tank and place this sample into a second clean plastic bottle labeled "LA Fiber Suspension Test: Sample C".
5. Promptly transmit all three bottles to the Libby on-site laboratory for analysis of LA. Both samples will be counted using TEM until a minimum of 100 LA structures have been enumerated in each sample. This will allow more than a 90% probability of detecting a relative percent difference (RPD) of 50%between the top and the bottom of the tank.
6. If no statistically significant difference is detected, tests with fish may begin. If a significant difference is detected, then alternative methods for ensuring that LA fibers are well mixed throughout the tank will be investigated before testing begins.

Please INSERT sampling for LA conc. of the dilution series as we have discussed..

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Comment [DW2]: I recognize that this is time consuming. These samples will need a quicker turnaround. Do we really need to have the chemical composition, sketches and all the other information required of a "standard" sample? We will get this information from our sampling of the dilution without the time pressure.

Feeding

No feeding will occur during the larval stage.

After swim-up occurs, fish will be fed freshly-hatched brine shrimp (about 12 hours post hatch) daily at a rate of 0.05 grams of brine shrimp per gram of fish in the aquariums. The average mass of each fish as a function of time may be estimated from measurements on fish grown in parallel tanks, or from historical growth curves.

Endpoints

Behavior

All aquaria will be observed daily at a consistent time for indications of differences in behavior between control fish and fish exposed to site waters. This may include, for example, differences in the frequency and duration of swimming events of the larvae, swimming and feeding behavior of the fry, etc. These observations will be recorded using the behavioral observation log sheet provided as Table 7-1.

Mortality

Observations on mortality will be recorded twice daily at approximately 8:30 AM and 4:30 PM. Table 7-2 2 is a form that will be used for assigning a unique identifies to each fish and for recording date and time of death of each fish.

Histopathology

All fish that die during the study and all fish alive at the end of the study will be preserved by being placed into fixative solution for subsequent histopathological evaluation. Detailed SOPs for sample preservation, slide preparation, and histological examination will be provided by the histological laboratory. Based on the work of Belanger (1985), it is expected that relevant endpoints may include dermal thickening and abrasion, abrasion or lesions of the gill, as well as lesions of the kidneys and GI tract. However, the histologist should seek to identify any potentially meaningful changes that appear to be treatment-related.

Growth

No measures of growth will be performed during the larval stage. After swim-up, measures of growth will include length and mass of the fish. Data on growth will be recorded using the form provided as Table 7-2.

Comment [DW3]: Why would you not weigh 15 "sacrificial" larvae at the initiation of the test? As written, I would run a tank of laboratory water in addition to the other tanks. 15 fish will be weighed when they reach swim-up. This will serve as the "initial swim-up weight" for all samples.

7.4 Data Reporting

For each water sample tested, the laboratory shall record data using the data sheets provided in Tables 7-1 and 7-2. (EPA will provide the laboratory with electronic copies of these tables to facility data entry and transmittal). The laboratory shall also provide a text report in which the conditions of the test and any deviations from the study protocol or any other issues are described and evaluated.

TABLE 7-1. BEHAVIORAL LOG

Water Sample = _____ Study Start Date = _____

Day	Observations
1	
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3	
4	
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13	
14	
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TABLE 7-2. MORTALITY AND GROWTH LOG

Test water description = **Mill Pond**
Test water code = **MP**

Aquarium	Fish	Label Assigned	Day of death	Time death was noted	Length (mm)	Weight (mg)	Notes
A	1	MP-A1					
	2	MP-A2					
	3	MP-A3					
	4	MP-A4					
	5	MP-A5					
	6	MP-A6					
	7	MP-A7					
	8	MP-A8					
	9	MP-A9					
	10	MP-A10					
	11	MP-A11					
	12	MP-A12					
	13	MP-A13					
	14	MP-A14					
	15	MP-A15					
B	1	MP-B1					
	2	MP-B2					
	3	MP-B3					
	4	MP-B4					
	5	MP-B5					
	6	MP-B6					
	7	MP-B7					
	8	MP-B8					
	9	MP-B9					
	10	MP-B10					
	11	MP-B11					
	12	MP-B12					
	13	MP-B13					
	14	MP-B14					
	15	MP-B15					
C	1	MP-C1					
	2	MP-C2					
	3	MP-C3					
	4	MP-C4					
	5	MP-C5					
	6	MP-C6					
	7	MP-C7					
	8	MP-C8					
	9	MP-C9					
	10	MP-C10					
	11	MP-C11					
	12	MP-C12					
	13	MP-C13					
	14	MP-C14					
	15	MP-C15					